



CHLORIDE EXCHANGES IN RAINBOW TROUT (*SALMO GAIRDNERI*) ADAPTED TO DIFFERENT SALINITIES¹

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The water and salt balance mechanisms used by teleost fishes have attracted considerable interest for almost a century (recent general reviews are: Black, 1957; Gordon, 1963; Prosser and Brown, 1961; Shaw, 1960). While a great deal of work has been done on fishes maintained under fairly constant conditions in either fresh or sea water, very little effort has been directed at studies of the changes in the basic regulatory mechanisms which occur when fishes encounter environmental changes. Salinity changes are among the commonest of these variations.

The responses of euryhaline teleosts are particularly interesting in this regard, since these forms are usually very good osmotic and ionic regulators. The relative constancy of the concentration of their internal medium means that, when these fishes enter environments of different salinities, they maintain across their integuments osmotic gradients of different magnitudes and even directions. These changes in osmotic gradients in turn mean that, in order for constancy of internal concentration to continue, the fishes must change either or both the fluxes and effective permeabilities for water and at least sodium and chloride across their major exchange pathways—the gills, gut and kidneys.

The three-spined stickleback (*Gasterosteus aculeatus*) seems to be the only euryhaline fish for which even limited data are available on the mechanism of adaptation to changes in trans-integumentary osmotic gradients. Heuts (1942) found that the chloride concentration in cloacal excreta increased about 20 times over fresh-water levels in sticklebacks in $\frac{1}{3}$ sea water. Mullins (1950) presented data which he interpreted as indicating that sticklebacks increased their rate of drinking of external medium as the concentration of that medium increased. He also thought that the permeability of the gills to specific ions, especially potassium, changed.

The rainbow trout (*Salmo gairdneri*) is a euryhaline teleost which survives well in both fresh water and the sea. It is a very good osmotic and ionic regulator (Busnel, 1942; Busnel and Drillhon, 1944, 1946; Houston, 1959, 1961; Parry, 1960, 1961). This paper describes a study of the rates of exchange of body chloride by rainbow trout acclimatized to fresh water and sea water, and to several intermediate salinities. The results are interpreted as indicating that the change-over from hyper-osmotic regulation in fresh water to hypo-osmotic regulation in sea

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water, at least in this species, is based in large part on changes in permeability to water of the gills and other permeable portions of the integument.

A preliminary discussion of some of these results was given by Gordon (1959a).

MATERIALS AND METHODS

Sexually mature two- to three-year-old rainbow trout, of 100–250 gm. weight, were obtained from a commercial fish hatchery. These fish were maintained in individual aquaria in the laboratory. Each aquarium was equipped with aeration and a system whereby circulating water of any salinity between fresh water (dechlorinated Los Angeles tap water) and 100% sea water (equals 32‰ salinity, 960 mOsmoles/liter osmotic concentration) could be supplied continuously for any desired period. Water temperature was maintained at $19 \pm 2^\circ$ C. Photoperiod was that normal for the season. No experiments were done during the autumn breeding season, when salinity tolerance in this species is sometimes markedly decreased. Fish were not fed.

Groups of fish were maintained in fresh water for several days after receipt. Ten days were allowed for acclimatization to each higher salinity used. Transfers were made directly from fresh water to $\frac{1}{7}$ and $\frac{1}{3}$ sea water. Acclimatization to still higher salinities was done stepwise (*e.g.* 10 days in $\frac{1}{3}$ sea water, then 10 days in $\frac{2}{3}$ sea water, then 10 days in $\frac{3}{3}$ sea water).

Estimates of rates of chloride exchange were carried out on groups of five trout acclimatized to fresh water, $\frac{1}{7}$, $\frac{1}{3}$, $\frac{2}{3}$, and $\frac{3}{3}$ sea water. Excepting only $\frac{1}{7}$ sea water, observations were made at each concentration, both on normal, intact fish and on fish which had had their cloacas and urinary papillae tightly ligated. The effectiveness of cloacal ligation was tested at the end of each experiment by pressing on the abdomen of the fish in order to extrude bladder urine or gut contents. In all cases pressures required were so far above those which the fish themselves could have produced that I am confident no leakage occurred during the experiments. All operations on trout were performed with the fish held under water in a piece of smooth cotton cloth. Water used was always that to which the fish were acclimatized.

Radioactive chlorine-36 was used as a tracer for chloride movements. This isotope was obtained as carrier-free HCl^{36} with specific activity of about 500 $\mu\text{c./gm.}$ chloride, from Oak Ridge. This solution was neutralized with NaOH and diluted to produce a final injection solution of about 160 mM NaCl^{36} . Final activities of injection solutions were in the range 20–30 $\mu\text{c./ml.}$

NaCl^{36} solutions were injected intraperitoneally. Total injection volumes were in the range 0.08–0.15 ml., calculated to produce doses of approximately 15 $\mu\text{c./kg.}$ The fish were then transferred to individual small closed plastic aquaria, each containing 1500 ml. of water of the appropriate concentration and supplied with aeration. Two-ml. aliquots of the external medium were taken at one-hour intervals for 6 hours. The fish were then sacrificed, a blood sample was taken by heart puncture, and they were re-weighed to the nearest gram.

The aliquots of external medium, also duplicate 25- $\mu\text{l.}$ aliquots of blood samples, were evaporated to dryness on aluminum planchets. Times required for the occurrence of 5120 counts per sample were determined with a Nuclear-Chicago Model D-47 thin end window (0.1 mg./cm.²) gas flow counter connected with an automatic

sample changer, scaler and printing timer. Background was stable at 15 cpm., resulting in assay precision of $\pm 2-3\%$. Absolute activity of samples was determined by comparison with standards of known activity made from the original HCl^{36} solutions obtained from Oak Ridge. These standards were dissolved in appropriate volumes of the sea water dilutions used, and prepared and counted in the same way as unknowns.

Total chloride in blood samples was determined on duplicate 0.1-ml. aliquots with an Aminco-Cotlove automatic chloride titrator. Precision was 2-3 meq./l. of whole, hemolyzed blood. Blood specific activity for each fish at the end of each experiment was calculated from these data and the radioactivity assays.

Measured rates of appearance of Cl^{36} were converted to $\mu\text{c. Cl}^{36}/\text{kg. fish/hr.}$ for fish with a uniform specific activity of their blood one hour after injection of 0.50 $\mu\text{c. Cl}^{36}/\text{meq. total Cl in blood.}$ These adjustments were required since there were variations in radioactivity of body chloride in each fish in each experimental group, which resulted from errors in initial weighing and variations in amount of total body chloride.

Procedure for adjusting rate measurements was as follows: No recycling of radioactivity into the fish was assumed, as was a steady-state for the chloride content of the fish. The fraction of the injected dose which appeared in the medium during the 6 hours duration of the experiment was calculated. Those few fish were discarded which had exchanged more than about 20% of the injected dose (these probably had been damaged in handling), as were those with such low blood chloride specific activities that it was apparent the initial injection had not been successful. Rates of exchange of radioactivity were assumed to have been proportional to blood chloride specific activity. Blood chloride specific activity one hour after the initial injection was assumed to have been equal to: measured final specific activity/fraction of injected isotope dose remaining in fish at end of experiment. A factor was then calculated for each fish which would adjust the calculated blood specific activity one hour after the injection to 0.50 $\mu\text{c./meq. Cl.}$ The measured rates of appearance of radioactivity for each fish, in $\mu\text{c./kg./hr.}$, were then multiplied by this factor.

Data for the first hour following the injections were not used. Mean rates of appearance, also standard errors, for each of the remaining five hours of each experiment were calculated. Excepting only the experiments in fresh water, there were no statistically significant differences within the sets of five hourly means for each experiment.

Cumulative rates of isotope appearance for the five usable hours of each experiment were next calculated for each fish in each group. Means and standard errors for these cumulative rates were calculated and comparisons between the various groups made by analysis of variance.

As a check on the osmotic and ionic regulatory abilities of the rainbow trout used in this work, blood samples were taken by heart puncture from additional groups of animals, acclimatized to various salinities under the same conditions as the experimental animals. No samples were taken during the breeding season. Freezing point depressions were determined by the method of Ramsay and Brown (1955), with a precision of $\pm 0.02^\circ \text{C.}$ Chloride concentrations were determined on the automatic chloride titrator as described above.

RESULTS

Plasma osmotic and chloride concentrations in rainbow trout acclimatized to various salinities from fresh water to $\frac{3}{3}$ sea water are shown in Figure 1. No seasonal changes were noted. These results agree with those of the other workers cited in the introduction. Plasma chloride concentration closely parallels osmotic concentration. Regulation of these concentrations is not perfect, but it is quite good. Fish in fresh water maintain osmotic gradients across their integuments almost twice as large as those maintained by fish in $\frac{1}{7}$ sea water. These gradients act to move water into the animal. Fish in $\frac{3}{3}$ sea water maintain osmotic gradients, in the opposite direction, 30 to 50 times larger than those maintained by fish in $\frac{1}{3}$ sea water.

Table I and Figure 2 present the results of the adjusted measurements of cumulative rates of Cl^{36} appearance from variously acclimatized trout, both unoperated fish and those with ligated cloacas and urinary papillae ("ligated fish"), during the period February through May. Table II summarizes the results of the statistical analyses of these data. The measurements on ligated fish are interpreted as estimates of the rates of chloride exchange across only the gills and other permeable parts of the integument. The differences between these rates and those for unoperated animals are considered estimates of the rates of chloride loss by way of the gut and, especially, the kidneys.

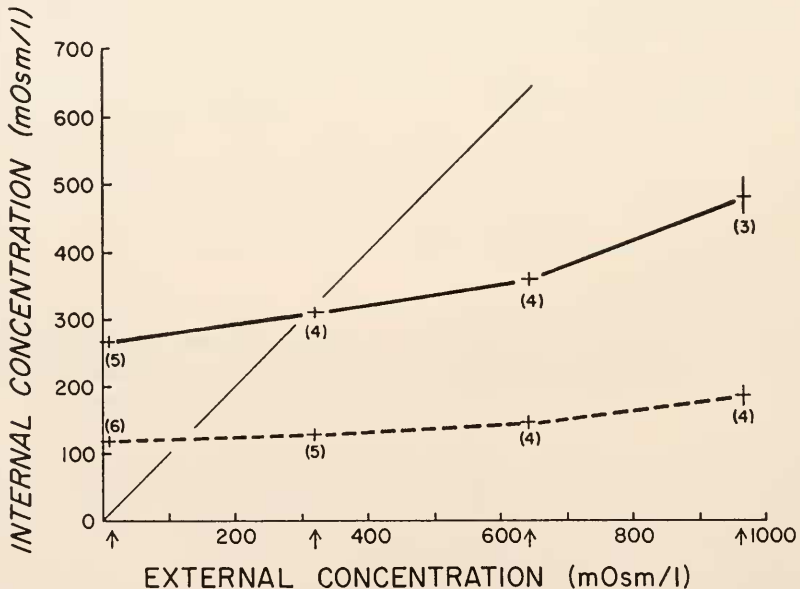


FIGURE 1. Osmotic and chloride concentrations of plasma vs. osmotic concentration of external medium, in rainbow trout acclimatized to various salinities. Horizontal lines indicate means of observations on indicated numbers of fish; vertical lines ± 2 S.E.'s. Solid line: plasma osmotic concentration (freezing point depression); dashed line: plasma chloride concentration. Diagonal line is line of equality. Fish acclimatized to each medium for at least 10 days. All experiments at $19 \pm 2^\circ \text{C}$. Arrows along abscissa indicate osmotic concentrations of fresh water, $\frac{1}{7}$, $\frac{1}{3}$ and $\frac{3}{3}$ sea water.

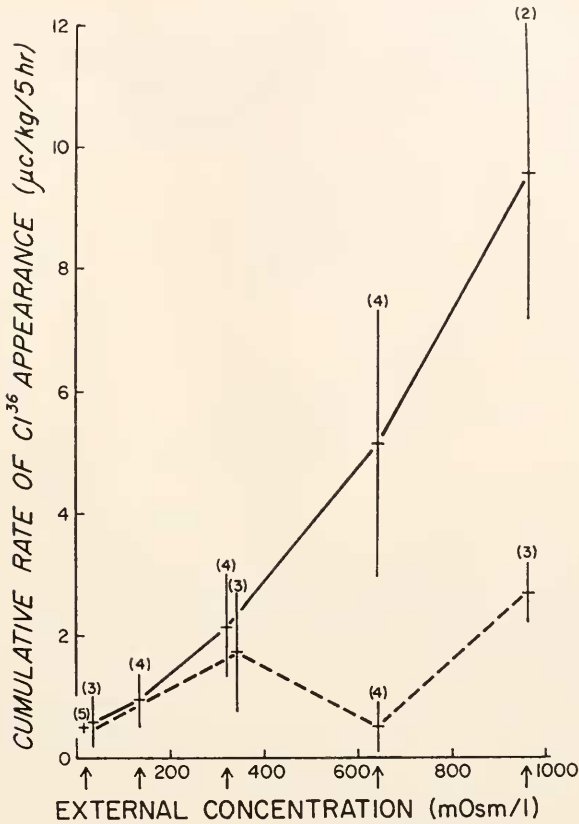


FIGURE 2. Cumulative rate of appearance of Cl^{36} from rainbow trout acclimatized to various salinities at $19 \pm 2^\circ \text{C}$. between February and May. See text for details. Solid line: unoperated fish; dashed line: fish with sewn cloacas and urinary papillae. Symbols as Figure 1.

Rates of total chloride exchange for unoperated animals in hypo-osmotic media (fresh water and $\frac{1}{7}$ sea water) are all statistically significantly lower than rates for unoperated animals in all of the hyper-osmotic media. Total exchange rates increased as external osmotic and chloride concentrations rose. Rates for fish in $\frac{2}{3}$ sea water are not statistically significantly different from rates for fish in $\frac{1}{3}$ sea water. The difference in rates between $\frac{2}{3}$ and $\frac{3}{3}$ sea water is significant.

Fish in fresh water are indicated to have exchanged chloride about one-half as rapidly as fish in $\frac{1}{7}$ sea water. The external chloride pool in the fresh-water experiments was sufficiently small so that this result may have been affected by recycling of isotope by the fish. There was a statistically significant continuous secular decline in rate of change of external isotope concentration in the fresh-water experiments. The rate of isotope appearance during the second hour of the fresh-water series was the same as the rate determined for the same period in $\frac{1}{7}$ sea water. The rate constants calculated for these two groups by analyses of the data as two compartment systems (Solomon, 1960, p. 130) were also the same. The true

TABLE I

Cumulative rates of Cl³⁶ appearance from rainbow trout (blood specific activity 0.50 µc. Cl³⁶/meq. Cl at one hour; 19 ± 2° C.)

State of acclimatization	Rates of Cl ³⁶ appearance (µc./kg./5 hr.) [$\bar{X} \pm S.E. (N)$]	
	February–May	July–August
<i>Unoperated fish</i>		
FW	0.51 ± 0.06 (5)	—
1/7 SW	0.98 ± 0.24 (4)	—
1/3 SW	2.16 ± 0.42 (4)	1.75 ± 0.16 (4)
2/3 SW	5.16 ± 1.09 (4)	3.68 ± 0.59 (4)
3/3 SW	9.6 ± 1.2 (2)	2.24 ± 0.17 (3)
<i>Fish with ligated cloaca and urinary papilla</i>		
FW	0.61 ± 0.22 (3)	—
1/3 SW	1.75 ± 0.48 (3)	—
2/3 SW	0.54 ± 0.20 (4)	—
3/3 SW	2.72 ± 0.24 (3)	—

cumulative rate for fresh water is therefore probably similar to the rate for $\frac{1}{4}$ sea water.

Fish in $\frac{3}{8}$ sea water exchanged chloride approximately $4\frac{1}{2}$ times more rapidly than fish in $\frac{1}{4}$ sea water, about twice as rapidly as fish in $\frac{2}{8}$ sea water.

The pattern for ligated fish is statistically significantly different from that for unoperated fish only in $\frac{2}{8}$ and $\frac{3}{8}$ sea water. The results for the ligated group in fresh water duplicated those for unoperated fish both in time sequence and cumulatively. This indicates that virtually all chloride exchange in trout in fresh water occurs across the gills and other permeable parts of the integument. The same appears to be true for fish in $\frac{1}{8}$ sea water.

The situation in strongly hyperosmotic media appears to be different. Rate of chloride exchange by ligated fish in $\frac{2}{8}$ sea water was only about 10% of that for unoperated fish. In $\frac{3}{8}$ sea water this fraction was near 25%. The rate for ligated fish in $\frac{2}{8}$ sea water is not statistically significantly lower than the rate for ligated fish in $\frac{1}{8}$ sea water. The rates for ligated fish in $\frac{2}{8}$ sea water and $\frac{3}{8}$ sea water differ significantly. The rates for ligated fish in $\frac{1}{8}$ sea water and $\frac{3}{8}$ sea water are

TABLE II

*"F" values resulting from analysis of variance comparisons between groups of rainbow trout, February through May**

Unoperated fish	FW	1/7 SW	1/3 SW	2/3 SW	3/3 SW	Ligated fish
3/3 SW	396***	297***	133***	13.0***	—	3/3 SW
2/3 SW	12.1***	9.59**	4.54	—	61.7***	2/3 SW
1/3 SW	12.4***	5.35**	—	2.50	1.40	1/3 SW
1/7 SW	3.95	—	—	—	—	1/7 SW
FW	—	—	2.20	0.30	55.6***	FW

*"F" values with no asterisks indicate that the groups compared are not statistically significantly different. Two asterisks indicate significance at the 5% level, three asterisks at the 1% level.

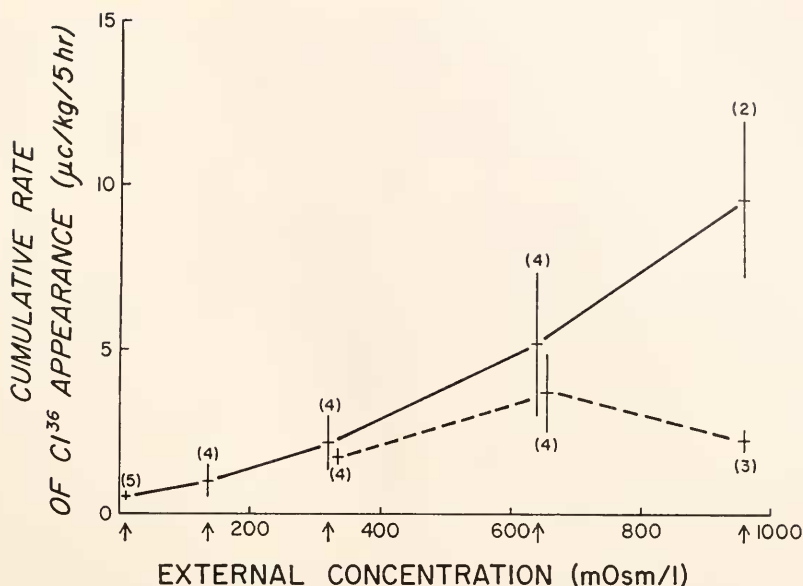


FIGURE 3. As Figure 2, but unoperated fish at different times of year. Solid line: experiments between February and May; dashed line: experiments in July and August.

not statistically significantly different. These results indicate that trout in hyperosmotic media may exchange by way of the permeable parts of their integument only a small fraction of the total amount of chloride they take in by drinking of external medium. The amount of chloride exchanged across these permeable areas (presumably primarily the gills) does not change greatly, even though the osmotic gradients maintained by the fish increase tremendously.

Table I and Figure 3 present the results of the adjusted measurements of cumulative rates of Cl^{36} appearance from variously acclimatized unoperated trout during the periods February through May and July through August. A comparison of these two periods was made because Gordon (1959b) had found the osmoregulatory abilities of the brown trout (*Salmo trutta*) to be significantly lessened during July and August, as compared with the rest of the year.

The July-August results differ from the February-May results only for fish in $\frac{3}{8}$ sea water. Trout acclimatized to $\frac{3}{8}$ sea water in summer exchanged chloride only $\frac{1}{4}$ as rapidly as similarly treated fish in spring. The lack of similar differences for trout in $\frac{1}{8}$ and $\frac{2}{8}$ sea water makes it improbable that this single difference actually reflects seasonal changes in the fish. It seems much more probable that the difference was due to differences in experimental manipulation of the two groups, the summer group having been less shocked and damaged by handling than the spring group.

DISCUSSION

Two major difficulties complicate the interpretation of the results of experiments such as these. First, fishes are notoriously sensitive to handling. This is due not only to the changes in internal hormonal concentrations resulting from the

stress imposed by experimental manipulations, but also to mechanical changes in the permeability of their integument due to rubbing off of mucus, scratches, etc. The usual result of these effects is the induction, apparently only in fish in hyper-osmotic media, of a more or less severe state of "laboratory diuresis." This condition is characterized by the production of abnormally large volumes of urine of abnormally high salt content. The condition appears almost immediately after handling of fish (Forster and Berglund, 1956; Holmes, 1961).

It is probable that the increased rates of chloride exchange shown by unoperated rainbow trout in $\frac{2}{3}$ and $\frac{3}{3}$ sea water were due in large part to the occurrence of progressively more severe cases of laboratory diuresis. The considerable variability of most of the measurements for such groups fits in with this interpretation. The low result for the group in $\frac{3}{3}$ sea water in summer is probably the most reliable of the four presented. It seems most reasonable, therefore, to consider all of these results as upper limits for the total rates of chloride exchange by the fish involved.

The second complication is that an unknown and perhaps variable fraction of the measured rates of isotope appearance may have been due to the physical exchange of unlabelled for labelled Cl atoms ("exchange diffusion") and not have represented actual unidirectional ionic fluxes produced by particular transport processes. None of the data required for estimation of the rates at which exchange diffusion may have taken place are available (Cooperstein and Hogben, 1959). It is therefore impossible to say with certainty that the rates measured for ligated fish represent either (a) rates of passive outward diffusion of chloride across the gills, etc., of trout in hypo-osmotic media, or (b) rates of active excretion of chloride across the same tissues of fish in hyper-osmotic media. The rates determined are, however, upper limits for the rates at which these processes could occur.

Even with these qualifications, several inferences still seem reasonable. Note again that in both hypo-osmotic and hyper-osmotic media neither total chloride exchanges nor integumentary chloride exchanges varied proportionally with the magnitudes of the trans-integumentary osmotic gradients maintained by the fish.

Assume that the surface/volume ratio for the trout is constant in all salinities. Let the null hypothesis be that the diffusion coefficients for water across the permeable parts of the integument of rainbow trout are constant in all media. Increases in magnitude of osmotic gradients should, in this situation, produce proportional increases in rates of water movement. Assuming the experimental fish were in steady states with regard to water and salt, the implications would be: (a) the more dilute the external hypo-osmotic medium, the greater the rate of urine production and, with fairly constant urinary salt content such as usually occurs in fishes, the more rapid the rate of urinary salt loss; (b) the more concentrated the external hyper-osmotic medium, the greater the rate of drinking of that medium and the more rapid the rate of active salt excretion by the gills (osmotic gradients maintained by trout in $\frac{3}{3}$ sea water were a minimum of 30 times larger than gradients maintained by fish in $\frac{1}{3}$ sea water; there is three times as much salt per unit volume in $\frac{3}{3}$ sea water as there is in $\frac{1}{3}$ sea water; trout in $\frac{3}{3}$ sea water might, therefore, be expected to excrete chloride 90 or more times faster than fish in $\frac{1}{3}$ sea water).

The data agree with neither of these predictions. It seems most improbable that the amount of integumentary exchange diffusion in hyper-osmotic media would

change sufficiently to mask changes in rates of active excretion of the magnitudes required by the model. It is probable, therefore, that a most important part of the process of salinity acclimatization in rainbow trout is a reduction in integumentary permeability to water. These permeability reductions are more or less proportional to the magnitudes of transintegumentary osmotic gradients, whatever the direction of these gradients.

This conclusion implies that adaptation to different salinities does not necessarily impose on rainbow trout markedly different energy requirements for osmoregulatory purposes. It is possible that much of the adjustment is taken care of by changes in the physical state of the permeable areas of the integument, changes mediated, perhaps, by a neurohypophysial hormone (Hays and Leaf, 1962).

Support for these results and inferences can be derived from some calculations. Assume that the cumulative rate of chloride exchange by trout in fresh water was actually that measured for fish in $\frac{1}{7}$ sea water. Assume also that the specific activity of the chloride exchanged was the same as the adjusted specific activity used for the blood, *i.e.*, $0.50 \mu\text{c. Cl}^{36}/\text{meq. total Cl}$. The total rate of chloride loss from trout in fresh water, on this basis, was about 9.6 meq. Cl/kg./24 hr. Krogh (1937) estimated that small rainbow trout in fresh water absorbed Cl from their environment at the rate of 7.2 meq. Cl/kg./24 hr. Phillips *et al.* (1958), working with small brook trout (*Salvelinus fontinalis*), measured a total rate of Cl uptake from fresh water of 3 mM Cl concentration of about 1.8 meq. Cl/kg./24 hr.

The data of Krogh (1937) and Holmes (1961) indicate that urinary Cl losses in fresh water should account for almost half of the total losses. The present data do not indicate this.

Other euryhaline species, such as the common eels (*Anguilla* spp.) drink 30–200 ml./kg./24 hr. when acclimatized to sea water (Smith, 1930; Keys, 1933). Assuming no exchange diffusion and a specific activity of body Cl of $0.50 \mu\text{c.}/\text{meq.}$, the maximum measured rate of total Cl exchange in $\frac{3}{3}$ sea water (February–May) is equivalent to the ingestion by the trout of about 200 ml./kg./24 hr. The rate for July–August fish is equivalent to about 40 ml./kg./24 hr.

Similar calculations for trout in $\frac{1}{3}$ and $\frac{2}{3}$ sea water give drinking rates of approximately 40 ml./kg./24 hr. and 100 ml./kg./24 hr., respectively, between February and May. Summer fish are similar.

SUMMARY

1. Estimates of rates of exchange of body chloride, both total exchanges and exchanges across the integument and by way of the gut and kidneys, have been made in rainbow trout (*Salmo gairdneri*) acclimatized to various salinities between fresh water and sea water (salinity 32‰). Radioactive chlorine-36 was used as a tracer of chloride movements.

2. Neither total Cl exchanges nor integumentary exchanges varied in proportion with changes in the magnitude of the transintegumentary osmotic gradients maintained by the fish. This result is interpreted as indicating that changes in the permeability to water of the integument (probably primarily the gills) are an important part of the salinity adaptation process in rainbow trout.

3. Laboratory diuresis and exchange diffusion of chloride are discussed as possible complications in this interpretation.

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